A study of biofilm forming organisms commonly associated with dentures

PhD. Thesis Synopsis and Abstract

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ABSTRACT

A study was carried out on biofilm forming organisms commonly associated with dentures and palatal surface of dentate subjects from Sullia town of South Canara, Karnataka. As an outcome, high prevalence of *Candida, Klebsiella* and *Lactobacillus* was observed. Microbial succession was seen in the colonization by different groups of microorganisms in denture wearers than non denture wearers, indicating that dentures are reservoirs of microbes. Though lactobacilli are relatively less pathogenic, the acid produced by them during carbohydrate fermentation attracts *Candida* and other acid loving microbes and enhances denture plaque formation. Presence of *Klebsiella*, being an uncommon oral flora, is a significant potential pathogen. Dentures being inert and non shading surfaces, especially self cure polymethacrylic resin, provided an ideal niche for the biofilm formation by oral flora. *Klebsiella* and *Lactobacillus* isolates did not show noticeable difference in their antibiotic sensitivity profile between denture wearers and dentate subjects. Amphotericin B, fluconazole and nystatin were the effective antifungal agents against *C. albicans* which was the predominant denture colonizer.

Candida, Klebsiella and *Lactobacillus* isolates were positive by PCR for the gene related to adhesion and biofilm formation. As the oral environment provides a constant source of nutrients for the oral flora the establishment of these microbes in the oral surface is to be expected. *Candida* isolates showed good biofilm formation in high sucrose containing nutrient media, Sabouraud's dextrose broth. Thus adequate cleansing after consuming sucrose rich diet can prevent *Candida* colonization. Biofilm formation was reduced in dilute medium among all the groups of microbes studied while in non diluted menstrua, there was formation of strong biofilm.

The result demonstrates the need for good denture and oral hygiene by cleaning after every meal and night removal of the denture. This reduces the biofilm forming denture flora and subsequent plaque formation. Most of the oral microbes are opportunistic pathogens and many systemic diseases are thought to originate from oral infections. Poor denture hygiene leads to accumulating plaque followed by malodour, denture stomatitis and increased susceptibility to oral infections mainly in elderly people. Thus, denture wearers, though free from oral disorders and lesions, are asymptomatic carriers of potentially pathogenic microbes.

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Synopsis

Introduction

Human oral flora is highly diverse with approximately 700 bacterial species, majority of which have not yet been cultured *in vitro*. The oral flora comprises a varied group of microorganisms including bacteria, fungi, mycoplasmas, and viruses but bacteria predominate. Several studies have shown that among Gram positive cocci, Gram positive bacilli, Gram negative rods and yeast are the predominant denture microflora.

Oral cavity presents numerous surfaces for microbial colonization. Dentures too attract oral flora easily due to its non – shading, porous nature and constant usage. Tissue contacting surface of the denture being less disturbed and relatively stagnant, enhances easy colonization of microbes especially acidogenic bacteria and *Candida*. This association gradually gets transformed into multispecies biofilm and later as denture plaque.

Denture plaque has not been studied to the same extent as dental plaque. Many denture associated infections emerge by the ability of oral microbes to adhere, colonize and form biofilm on the fitting surfaces of the dentures. Several environmental factors like, pH, temperature of the contact surface, flow rate of the fluid passing over the surface nutrient availability, contact time of the bacteria with the surface, the growth stage, surface hydrophobicity are some of the factors that affect the bacterial attachment in biofilm formation.

Oral bacteria have been implicated in bacterial endocarditis, aspiration pneumonia, gastrointestinal infection and chronic obstructive pulmonary disease, and dentures offer a reservoir of potentially pathogenic microorganisms associated with these infections. Thus this study will help us to provide insights into the differential ability of organisms to form biofilms and molecular detection of virulence genes that aid biofilm formation and their resistance. To fulfill the above requirements, microbial composition of the removable maxillary dentures was studied compared to those with own dentition with following objectives.

Objectives

1. To isolate the predominant, aerobic biofilm forming organisms from the dentures.
2. To study the biofilm formation by predominant isolates by microtitre plate assay.
3. To understand the antibiotic susceptibility pattern of microbial isolates.
4. To study the prevalence of *Candida* colonization on dentures of complete maxillary denture wearers by conventional method.
5. To study the factors responsible for the adhesion or colonization and to check the presence of virulence genes in biofilm forming organisms using molecular methods.

1. Isolation of predominant, aerobic microbes from the denture wearers

Methodology: The samples for this study were from adult denture wearers (DW) and dentate or no denture wearers (NDW) subjects including, both male and female in a age group of 31 to 80 years from Sullia, town in Dakshina Kannada district, Karnataka. Various inclusion and exclusion criteria were followed and documented the patient profile.

The most problematic denture plaque occurs on the maxillary fitting surface, hence the palate facing surface of the denture was considered for isolation of microbes in our study. Using a sterile cotton swab, the palate facing surface of the dentures were swabbed thoroughly and processed within 1 hour on common selective media for microbiological examination. Every swab was processed on three selective media MacConkeys agar (MCA), Mann Rogosa Sharpe agar (RA) and Sabouraud's agar with gentamycin 2 mg/dL and chloramphenicol 5 mg/dL (SDA) (HiMedia, Mumbai.). The plates were assessed by semi-quantitative standard loop method. Representative colonies from MCA, SDA and RA plates were picked and subjected to a series of biochemical tests for identification of the bacteria. Morphology of the isolates was studied by Gram staining and lactophenol cotton blue staining. Identification of *Candida* was done by studying surface growth in SD broth, Carbohydrate assimilation test, Carbohydrate fermentation test, serum germ tube test and studying chlamydospore formation on Corn meal agar. Standard procedures were applied for the microbial culture and identification. Data generated was analyzed using student's'‘t' and 'chi-square' test.

Result: Being from healthy subjects, denture samples have shown significantly higher prevalence ($p<0.001$) and diversity of isolates. Out of the 50 samples in the DW group, the

highest prevalence (100%) was seen for yeast belonging to *Candida* species, followed by bacteria belonging to *Klebsiella* (78%) and *Lactobacillus* (72%) species. In contrast, in the NDW (n=50) the prevalence for *Candida*, *Lactobacillus* and *Klebsiella* species was observed to be lower having a prevalence percentage of 52%, 36% and 34% respectively. In DW, the commonest of *Candida* species amongst the *Candida* isolates were *C. albicans* (58%) followed by *C. tropicalis* (28%), *C. dubliniensis* (12%) and *C. glabrata* (2%) while in control group there were only *C. albicans* (50%) and *C. tropicalis* (2%) with 52 % of prevalence ($p<0.001$).

Lactobacillus species from the denture wearers showed more diversity in the species (*L. casei*, (40%), *L. plantarum* (24%), *L. fermentum* (2%), *L. salivarius* (2%), *L. brevis* (2%), *L. acidophilus* (2%) than non denture wearers where *L. casei* (33%), *L. plantarum* (33%) ,*L. fermentum* (23%), *L. salivarius* (11%) were the only species. Among *Klebsiella* isolates, *K. pneumonia* (82% and 94%) and *K. oxytoca* (18% and 6 %) were the only species in denture wearers and non denture wearers respectively. *E. coli, staphylococci, sterptococci, Serratia* and *Pseudomonas* species were the less prevalent isolates found in the study.

The study showed that there is significant difference ($p<0.001$) in the prevalence of denture and palatal isolates of *Lactobacillus, Klebsiella* and *Candida.* However, the prevalence and distribution of *Lactobacillus* species was different in both the groups with high significant difference (*X2=16.176, p< 0.001*). Whereas in case of *Klebsiella*, though the percentage of occurrence in both the groups was different, there were only two common species i.e. *K. pneumonia* and *K. oxytoca.* ($x^2=19.642$, $p< 0.001$).

2. Biofilm formation by the *Klebsiella, Lactobacillus* and *Candida* species from the dentures.

Methodology: Among the denture isolates, *Klebsiella* and lactobacilli were the predominant bacteria. Hence, they were considered for the biofilm study. Biofilm production by all the *Klebsiella* isolates from denture wearers and non denture wearers was carried out and quantified in 96 well flat-bottomed polystyrene microtiter plate with 1:10 and 1:100 Luria Bertani broth (LBB). For *Lactobacillus* isolates 1/10 MRS (Man Rogosa Sharpe) and 1/100 MRS was used for the biofilm formation. The method described by Stepanovic *et al.*(2000) for staphylococci biofilms was followed with some modifications for the biofilm study of *Klebsiella* and *Lactobacillus* spp. Based on the absorbance values measured at 630 nm (OD_{630}) in an ELISA

reader (ELx 800, Bio-Tek, 100 Tigan Street, Winooski, VT05404, USA) the isolates were classified as strong, moderate, weak and non-biofilm former.

1) Biofilm formation by *Klebsiella* isolates.

Result- In denture wearers, *Klebsiella* species showed only medium and strong biofilms in 1/10 and 1/100 LB broth. Dilution of the medium has increased weak biofilm formers than strong biofilm formers. In case of *K. pneumonia* when their biofilm formation in these 2 different media were compared, in 1:100 LBB, the number of medium biofilm formers have been increased from 32 % in 1/10 LBB to 46% . On the other hand strong biofilm formers have been decreased from 69% in 1/10 LBB to 36% in 1/100 LBB. *K. oxytoca also* showed only medium and strong biofilm formation in denture wearers in both the dilutions of LBB. In 1/10 LBB, *K. oxytoca* showed highest moderate biofilm (86%) formers whereas in 1/100 LBB, number of strong biofilm formers were increased from 14 % in 1/10 LBB to 57% and moderate biofilm formers were decreased from 86% in 1/10 LBB to 43%. Result shows that dilution of the nutrients has encouraged more medium biofilm formers than strong biofilm formers in *Klebsiella* isolates.

Among non-denture wearers *K. pneumonia*, showed weak (41%), medium (41%) and strong (6%) biofilm formation. Non biofilm formers were 12 % and 6% in both 1:10 and 1:100 LBB respectively. While in 1:100 dilutions of LBB, strong biofilm formers have remained same i.e. 6%. In 1/100 LBB moderate biofilm formers were increased from 36% in 1:10 LBB to 56%. This shows that *K. pneumonia* from non denture wearers showed increased biofilm formation in more diluted medium. Thus *K. oxytoca* in both test and control group showed good biofilm formation irrespective of nutrient dilution.

2) Biofilm formation by *Lactobacillus* isolates

Result-Out of 36 *Lactobacillus* isolates from denture wearers including six species, there were weak (25%) and medium (75%) biofilm formers in 1:10 dilution of MRSB while in 1:100 MRSB only medium (41%) and no (59%) biofilm formers were seen. In DW, varied biofilm formation was observed. Six *L. casei* showed weak (10%) and medium (90%) biofilm in 1/10 MRSB while in 1/100 MRSB number of medium biofilm were reduced and weak biofilms were increased (55%). Six *L. plantarum* showed 92% biofilm formation and only 8% of weak biofilm

in 1:10 MRSB whereas in 1:100 MRSB there were no medium biofilm but 50% each of no and weak biofilm formers were observed. One *L. fermentum* which was weak biofilm former lost it's adhering ability, showing no biofilm in 1:100 MRSB. One *L. salivarius* with medium adhering ability in 1:10 MRSB also lost it's adherance in 1/100 MRSB. *L. brevis* and *L. acidophilus* showed weak biofilm formation in 1:10 while their adhering ability was nullified in 1/100 MRSB. Thus biofilm formation of *L. fermentum, L. salivarius, L. brevis* and *L. acidophilus* was found to be drastically affected by diluted nutrients among denture wearers.

Eighteen *Lactobacillus* isolates comprising four species from NDW showed weak (78%), medium (11%) and 11% of no biofilm formers. Among them, six *L. casei* showed medium biofilms in 1/10 MRSB which was found reduced to 5 with one weak biofilm former in 1/100 MRSB. Out of the six *L. plantarum* 4 were medium and 2 were strong biofilm formers in both the dilutions of MRSB. Four medium biofilm forming *L. fermentum* in 1/10 MRSB showed weak biofilm in 1/100 MRSB. Two medium biofilm forming *L. salivarius* isolates showed reduced adhering ability in 1/100 MRSB with only one weak biofilm former and one no biofilm former.

3) Biofilm formation by *Candida* isolates

Methodology-The adherence capacity of *Candida* isolates from denture wearers (n=50) and non-denture wearers (n=26) were assessed by using 96 well microtitre plate in 1:10 and 1:100 dilutions of Yeast Nitrogenous base broth (YNBB), (Hi Media, Mumbai). Protocol for the inoculum preparation and biofilm formation for *Candida* isolates followed those of Chandra *et al.*, (2001) and Ramage *et al.*, (2001) with few modifications.

Candida isolates were inoculated in SDB for overnight. One reference strain of *C. albicans* ATCC 24433 was used for identification. The optical density of the wells was observed in ELISA X 800 readers at 570 nm. Based on the O.D. produced by *Candida*, biofilms were quantified by a method same as that of *Lactobacillus* and *Klebsiella.*

Result-Out of all the *Candida* isolates grown in 1:10 YNBB, all the 14, *C. tropicalis*, (29%-weak, 71%- moderate biofilm formers) were biofilm formers. *C. glabrata* (1) showed moderate biofilm formation. *C. dubliniensis* showed all the four categories of biofilm formation namely strong (16%), Moderate (16%), weak (34%) and no biofilm (34%). Out of the 29

C. albicans, only 41% were biofilm formers in 1/10 YNBB and in 1:100 YNBB, weak biofilm formers increased from 10 to 20%. One *C. tropicalis* showed no biofilm formation. Among *C. dubliniensis*, number of non biofilm formers has doubled in 1:100 YNBB. Diluted YNB has not affected the biofilm formation of *C. glabrata.*

In case of NDW, non biofilm formers were more than biofilm formers (73% in 1:10 YNBB, 81% in 1:100 YNBB). Biofilm formers were only 27% and 19% respectively in 1:10 YNBB and in 1:100 YNBB. Thus denture wearers showed more pathogenic potential through biofilm formation than non denture wearers.

3. Antibiotic sensitivity pattern of the denture isolates.

a) Antimicrobial susceptibility profile of *Klebsiella isolates*-

Methodology - The antimicrobial susceptibility of *Klebsiella* isolates from DW (n=39) and NDW (n=17) were tested by standard agar disc diffusion technique (Bauer *et al*., 1966) on Mueller Hinton agar using commercial discs (Hi-Media, Mumbai, India). Twelve antibiotics were used for the assay, they were ampicillin, erythromycin, cephalexin, chloramphenicol, gentamycin, kanamycin, penicillin G, Co-trimoxazole, novobiocin, cloxacillin, norfloxacin and cefuroxime. The isolates were grown in MRS broth for 16-18 hr and adjusted culture concentration to 0.5 McFarland's to use as inoculum. Rest of the procedure for the antibiotic sensitivity of *Klebsiella* was same as that of lactobacilli. Results were recorded by measuring the inhibition zones, compared with the interpretative chart and classified according to the patterns of antibiotic sensitivity.

Result-All the *Klebsella* isolates from denture wearers showed resistance to ampicillin (91%), erythromcin (95%), penicillin (98%), cephalexin (82%), cloxacillin (90%), cefuroxime (90%) and complete resistance for novobiocin. Highest sensitivity was shown for gentamycin (93%), norfloxacin (89%), and kanamycin (75%) and for co-trimoxazole, it was 66%. Intermediate sensitivity was found for kanamycin (18%) and norfloxacin (9%) while or kanamycin, lowest intermediate sensitivity was seen in case of chloramphenicol, gentamycin, erythromycin cephalexin and cloxacillin. The non denture wearers also showed similar antibiogram.

b) Antimicrobial sensitivity profile of *Lactobacillus* isolates-

Methodology-The antimicrobial susceptibility of *Lactobacillus* isolates from DW (n=36) and NDW (n=18) were tested by standard agar disc diffusion technique (Bauer *et al*. 1966) on Mueller Hinton agar using commercial discs (Hi-Media, Mumbai, India). The antibiotics used were ampicillin, cloxacillin, cephalothin, chloramphenicol, ciprofloxacin, gentamycin, penicillin G, rifampicin, tetracyclin, kanamycin erythromycin, amicacin, co-trimoxazole, nitrofuran and norfloxacin. The isolated 16-18 hr old cultures in Luria Bertani broth with the concentration of 0.5 Mc Farland's concentration were used.

Result- Results of sensitivity patterns were recorded by measuring the inhibition zones. Among the *Lactobacillus* isolates of denture wearers, complete sensitivity was shown to tetracyclin, cloxacillin, ampicillin, chloramphenicol, while complete resistance was shown to ciprofloxacin and Kanamycin. Remaining *Lactobacillus* isolates showed lesser sensitivity to eryhromycin (10%), penicillin (45%), gentamycin (9%), novobiocin (9%), co-trimoxazole (9%), and norfloxacin (9%) while higher sensitivity was seen for rifampicin (82%) and nitrofuran (73%).

Isolates from the control group were found complete sensitive to tetracycline, cloxacillin, ampicillin, chloramphenicol, penicillin and nitrofuran while complete resistance was shown to ciprofloxacin, kanamycin, erythromycin gentamycin, novobiocin, co-trimoxazole, rifampicin and norfloxacin with only 50% sensitivity. The biofilm formation and antifungal and antibiotic sentitivity revealed no definite correlation.

c) Antifungal sensitivity of *Candida* isolates-

Methodology-The antifungal susceptibility pattern of *Candida* isolates was done by standard agar disc diffusion technique on modified Mueller Hinton agar (with 2% glucose and 0.5% mcg/ml of Methylene blue) using commercial discs (Hi-Media, Mumbai, India). Antifungal discs of amphotericin B, clotrimaxazole, fluconazole and nystatin were used. The isolated 16-18 hr old *Candida* cultures with the concentration of 0.5 Mc. Farland's standard (1.5×10^{8} cells /ml), were used as inoculum. Remaining procedure and recording the results for the antifungal sensitivity of *Candida* isolates was same as that of *Klebsiella* and *Lactobacillus*.

Result-Antifungal sensitivity of 50 *Candida* isolates showed varying susceptibility. *C. glabrata* and *C. dubliniensis* were less prevalent and showed 100% sensitivity to amphotericin B and nystatin while 100% resistance was shown to clotrimazole and 50% resistance to fluconazole. *C.*

albicans was highly prevalent and was found more sensitive to amphotericin B (95%), fluconazole (80%) and nystatin (95%). The highest loss of sensitivity (49%) was found for clotrimazole. *C. tropicalis* was sensitive to amphotericin B (79%), fluconazole (65%) and nystatin (86%) and 65% resistant was shown to clotrimazole. Overall all the *Candida* isolates have shown (56%) resistance to clotrimazole and significant difference was found in the sensitivity towards fluconazole, amphotericin B and nystatin ($p<0.001$) compared to clotrimazole. In NDW, there were only *C. albicans*, (50%) and *C. tropicalis* (2%) isolates. *C. albicans* was found sensitive to amphotericin B (100%), fluconazole (96%) and nystatin (96%) while 32% resistance was seen towards clotrimazole. On the other hand *C. tropicalis* has shown more sensitivity towards all the antifungal agents used in the assay.

4. Prevalence of *Candida* Colonization among denture wearers.

For the prevalence study of *Candida* colonization, total 100 denture samples were considered. Inclusion, exclusion criteria and procedure was similar to that of first objective. The growth of *Candida* was assessed by semi quantitative method on SDA. The result showed that there was 100% prevalence of *Candida* in all the denture wearers. Biochemical tests for the identification of *Candia* shows that, out of 100 Candida isolates, *C. albicans* were the highly prevalent (53%) species. *C. tropicalis* were the second most prevalent species (28%), *C. dubliniensis* were the third prevalent (13%) species. Prevalence of *C. krusei* was 4% and that of *C. glabrarta* was 2%. This data suggest that dentures are the ideal sites for multispecies *Candida* colonization.

5. Study of factors responsible for the biofilm formation.

Biofilm formation of all the *Klebsiella* isolates in 1:10 and 1:100 LBB and *Lactobacillus* isolates in 1:10 and 1:100 MRSB using 96 well polystyrene microtitre plate has been already studied in the second objective.

1. **Mixed species biofilm formation by *Lactobacillus* species.**

Methodology-In addition to the study of biofilm mentioned earlier, lactobacilli isolates were also investigated for their biofilm forming ability under two nutrient conditions, normal strength

Mann Rogosa Sharpe broth (MRSB) and 1/10 diluted MRSB by polystyrene microtitre plate assay. Six representative *Lactobacillus* isolates from dentures that included *L. fermentum, L. plantarum, L. casei, L. salivarius, L. brevis,* and *L. acidophilus* were studied for biofilm forming ability in pure and mixed culture conditions. For the mixed culture biofilm study, an aliquot of 10 µl of each of the two *Lactobacillus* species was added together to the wells as inocula. Every *Lactobacillus* isolate was mixed with each of the other five *Lactobacillus* isolates. Procedure for the biofilm formation and quantification of biofilm remained the same as that of monoculture described earlier.

Result-All the *Lactobacillus* isolates produced biofilm on polystyrene surface in both MRSB and 1:10 diluted MRSB. However, the degree of biofilm formation varied. *L. plantarum* was a strong biofilm former in pure culture but it could not form a strong biofilm with any of the *Lactobacillus* species in mixed culture in both high and low nutrient medium concentrations. This resulted in moderate to weak biofilms. *L. brevis* and *L. salivarius* were moderate biofilm formers in pure culture and formed strong biofilm in combination. Significant difference was found in their biofilm forming ability in MRSB and diluted MRSB ($P < 0.001$). Result suggests that in addition to nutrient concentration and nature of the surface, each member in a mixed species culture also has a vital role to enhance or suppress the biofilm formation.

2. Effect of different media and surfaces on the biofilm formation of *C. albicans.*

a) Biofilm formation by *Candida* isolates in artificial saliva, S**abouraud's agar, Brain heart infusion broth and Tryptone soya broth in 96 well polystyrene microtitre plate.**

Methodology- Three representative *Candida albicans* isolates who showed strong, weak and no biofilm in YNBB were further tested for their biofilm formation in four different nutrient media namely artificial saliva, (Sabouraud's agar, brain heart infusion broth and tryptone soya broth also, in 96 well polystyrene microtitre plate. Protocol for the inoculum preparation and biofilm formation for *Candida* isolates followed those of Chandra *et al.,* (2001) and Ramage *et al.*, (2001).

Result- The result of *C. albicans* biofilm in four media showed that strong and medium biofilm formers showed strong biofilm in SDB (Sabouraud's Dextrose Broth) and medium biofilm formation in remaining three media. Weak biofilm formers showed medium biofilm formation in

BHI (Brain Heart Infusion Broth) and TSB (Tryptone Soya broth) while weak biofilm was shown in AS (Artificial Saliva) and SDB whereas non biofilm formers did not show any biofilm in any of the tested media.

2. Biofilm formation by *C. albicans* on glass surface and denture materials

Methodology- In addition to the polystyrene surface and different dilutions of YNB, biofilm formation of *Candida* was also tested in four different media, on 18 mm glass coverslip and three types of denture materials. These same strong, weak and non biofilm formers of *C. albicans,* used above were assessed for their their biofilm formation on the on 18 mm glass coverslip kept inside the 24 well microtitre plate and denture materials (self cure polymethacrylic resin, heat cure polymethacrylic resin and Co-Ni alloy) using the same four media. Sterile glass coverslips were inserted in the 24 well microtitre plate with 20 µl *Candida* inocula and plates were incubated for 24 h. Biofilm formed on the glass coverslip by *Candida* isolates was assessed by staining it with Calcoflour white and KOH, under fluorescent microscope.

Result- a) The strong biofilm formers showed abundant growth with numerous pseudohyphae while weak biofilm formers showed numerous *Candida* cells with very few pseudohyphae and non biofilm formers showed no pseudohyphae but scanty growth.

b) The biofilm formation by the *Candida* isolates on the denture materials strip (2 cm x 3 cm) was enumerated by standard plate count in terms of cfu/ml. Highest biofilm formation by strong and medium biofilm formers was observed on self cure polyacrylic resin then on heat cure resin and least was observed on Co-Ni alloy. Non biofilm formers have not shown any biofilm formation on any of the denture materials in any of the four nutrient media.

In conclusion, *Candida albicans* showed better biofilm formation in SDB compared to AS, BHI and TSB and among the denture materials self cure polymethacrylic resin attracted more *Candida* cells in the form of biofilm due to its micro porous nature and dilution of the nutrients has reduced the ability to form biofilm. On polysterene surface and glass coverslip *Candida* showed correlating adhering ability under similar nutrient conditions.

6. Identification of virulent genes associated with biofilm formation by PCR.

A single virulent gene related to the biofilm formation was targeted in *Klebsiella, Lactobacillus* and *Candida* and identified by PCR.

1) *mrkA* gene in *Klebsiella* isolates

Genomic DNA from *Klebsiella* isolates was extracted according to the method of Ausubel and colleagues (1992). In *Klebsiella mrkA* a gene coding for mannose resistant *Klebsiella*'s agglutinin like fimbrial protein was selected. In *Klebsiella,* a primer was designed to detect a 615 base pair *mrkA* by PCR. Positive and negative controls were used for the reference. All the isolates showed the presence of *mrkA* gene which encodes for the fimbrial protein and biofilm. In all the isolates, the selected virulent gene was identified but it was not expressed in the form of biofilm by all the isolates.

2) *luxS* gene in *Lactobacillus* isolates-

Gene *luxS* (coding for biofilm formation and auto inducer-2, AI-2 in quorum sensing) was targeted. Shrimp DNA extraction method (Otta *et al*. 2003) was used for genomic DNA extraction in *Lactobacillus* isolates. In case of *Lactobacillus* isolates, a primer sequence common for all the *Lactobacillus* species was designed to detect a 173 base pair *luxS* gene by PCR. Positive and negative controls were used for the reference. Though *luxS* gene was present in all the species of *Lactobacillus* isolates of denture and non denture wearers, it was not expressed in the form of biofilm in all the *Lactobacillus* isolates.

3) *ALS1* gene in *Candida* isolates.

In *Candida* isolates *ALS1* (agglutinin like secretary protein) coding for adhesin was selected and identified by PCR. Hence a primer (Green C.B.*et al*., 2004) of 318 base pair for *ALS1* gene coding for agglutinin like sequences responsible for adhesion in biofilm formation, was selected out of the family of 9 *ALS* genes. An *ALS1* gene activity accompanies *C. albicans* pathogenesis in biofilm formation. The ALS proteins mediate adhesion to epithelia, colony formation and biofilm formation. Isolation of DNA from *Candida* isolates was done for PCR (Sambrook *et.al*.). All the *C. albicans* showed the presence of *ALS1* gene.

PCR for all the 3 isolates were carried out in 30 µl reaction mixture. The PCR products were detected by agarose gel electrophoresis and the bands were visualized under UV transilluminator (Herolab, Germany).

In a nutshell, it could be summarized that, higher prevalence of *Candida, Lactobacillus* and *Klebsiella* was found in denture wearers than non denture wearers, indicating that dentures are reservoirs of microbes. Though lactobacilli are found relatively harmless in complete denture wearers, presence of *Klebsiella,* being an uncommon oral flora, is of significance as a potential pathogen. Thus dentures being inert and non shading surfaces, especially self cure polyacrylic resin dentures provided an ideal niche for the biofilm formation of *Candida* isolates. *Klebsiella* and *Lactobacillus* isolates did not show noticeable difference in their antibiotic sensitivity profile between denture wearers and dentate subjects. Amphotericin B, fluconazole and nystatin were the effective antifungal agents against *C. albicans* which was the predominant denture colonizer.

Candida, Klebsiella and lactobacilli isolates showed the presence of gene related to adhesion and biofilm, as the oral environment provides the constant source of nutrients for the oral and denture flora. *Candida* isolates showed good biofilm formation in high sucrose containing nutrient media (SDB). Thus adequate cleansing after consuming sucrose rich diet can cut down *Candida* colonization. As biofilm was found reduced in diluted nutrients among these predominant isolates, it clearly suggests that keeping good denture and oral hygiene by rinsing after every meal and night removal of the denture, one can reduce the biofilm forming denture flora and subsequently plaque formation. If denture hygiene is not maintained properly accumulating plaque may also lead to malodor, denture stomatitis and increased susceptibility to oral infections mainly in elderly people. Thus denture wearers though free from any oral disorders and lesions, were found as the asymptomatic carriers of potentially pathogenic microbes.

Published /Accepted papers/Oral presentations in the scientific journals

1. **P. Bharathi,** P.P. Bhowmick, M. Shekar, I. Karunasagar **(2011),** Biofilm formation by pure and mixed culture of *Lactobacillus* isolates on polystyrene surface in varying nutrient conditions. Journal of Bioinformatics,Biotechnology and Bioengineering **(Published).**

2. **P. Bharathi,** Malathi Shekar, Indrani Karunasagar. Evaluation of antimicrobial activity of *Lactobacillus* species associated with dentures (2011), Journal of Bioinformatics, Biotechnology and Bioengineering **(Published).**

3. **P. Bharathi,** Maiti B, Karunasagar I., Molecular characterization of *luxS* in *Lactobacillus casei* from denture wearers. 3 rd International conference on 'Genetic and Molecular Diagnosis In Modern Medicine And Biology 2010. Yenepoya Medical College, Mangalore (**Oral Presentation).**

Published abstracts in conferences and symposium

1. **Bharathi Prakash**. Jyothi K., Karunasagar I., Kotigadde S. (2007)A study of *Candida* colonization on denture surfaces. National Conference of Indian Association of Medical Microbiologist. Mangalore

2. **Bharathi Prakash**. Patit Paban Bhowmick, I. Karunasagar. (2009) Effect of media and surfaces on the biofilm formation by *Candida* spp from denture plaque. (MM-027).50th Annual Conference of Microbiologist of India. National Chemical Laboratory. Pune.

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3. **Bharathi Prakash**. Patit Paban Bhowmick, I. Karunasagar. (2009) Prevalence and biofilm formation of *Klebsiella* from denture plaque.(MM-028). 50th Annual Conference of Microbiologist of India. National Chemical Laboratory. Pune.

DEPARTMENT OF FISHERY MICROBIOLOGY

COLLEGE OF FISHERIES, MANGALORE

Certificate

This is to certify that **Bharathi Prakash** has carried out all the research work on the topic **'A study of biofilm forming microbes commonly associated with dentures'** under my direct supervision. I further confirm that, 2 papers out of her research work have been published in scientific journal.

Date: 27.1.12

Place: Mangalore

Dr. (Mrs.) Indrani Karunasagar

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